Toxicity assessment and prediction of a realistic pesticide mixture from a Portuguese agricultural area using concentration-response surface statistics

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Resumo

Estudos anteriores mostraram a co-ocorrência do inseticida organofosforado clorpirifos e do herbicida s-triazina terbutilazina em águas de superfície de áreas agrícolas na "Lezíria do Tejo", Portugal. No presente estudo, foram examinados os efeitos destes pesticidas isoladamente ou como uma mistura binária sobre a imobilidade de *Daphnia magna* e sobre a taxa de crescimento da microalga *Pseudokirchneriella subcapitata*. Terbutilazina e clorpirifos quando expostos individualmente provocaram uma resposta muito tóxica ou tóxica, em ambos os organismos. Normalmente, a toxicidade de misturas é avaliada em relação aos modelos de referência Adição de Concentração (AC) e Ação Independente (AI). Numa fase inicial deste estudo, os dados foram ajustados aos dois modelos de referência para avaliar os efeitos combinados de clorpirifos e terbutilazina. Para o *endpoint* imobilidade, os dados ajustaram-se melhor ao modelo AI, como era esperado, uma vez que os pesticidas apresentam modos de ação diferentes, porém foi observado um padrão específico; em doses baixas a imobilidade foi inferior à modelada (antagonismo), enquanto com doses muito elevadas a imobilidade foi superior à modelada (sinergismo). Por outro lado, não foi observado desvio da ação independente no teste com a microalga. Este estudo representa um passo importante para entender as interações entre pesticidas detetados anteriormente em estudos de monitorização de campo em áreas agrícolas na "Lezíria do Tejo", Portugal. A toxicidade da mistura observada foi comparada com as previsões, calculadas a partir das funções de resposta da concentração de clorpirifos e terbutilazina em dois rácios de concentração realistas, aplicando os padrões biologicamente relevantes nos quais ocorreram desvios. As misturas realistas apresentaram previsões exatas, embora se tenha obtido uma pior previsão para a mistura de clorpirifos 0,17 e terbutilazina 85 µg/L.

Palavras-chave: Mistura, clorpirifos, terbutilazina, *Daphnia magna*, *Pseudokirchneriella subcapitata*, toxicidade

Abstract

Previous work showed the co-occurrence of the organophosphate chlorpyrifos and the s-triazine herbicide terbuthylazine in surface waters of agricultural areas in “Lezíria do Tejo”, Portugal. In the present study, we examine the effects of these pesticides singly and as a binary mixture on the immobility of *Daphnia magna* and on the growth rate of the microalgae *Pseudokirchneriella subcapitata*. Terbuthylazine and chlorpyrifos at single exposure caused a very toxic or toxic response in both organisms. Usually, the toxicity of mixtures is evaluated in relation to the reference models Concentration Addition (CA) and Independent Action (IA). Initially, in this study was used the CA and IA model was to evaluate the joint effects of chlorpyrifos and terbuthylazine. For immobility endpoint, the data fits better to the IA model, due to different mode of action of the pesticides, however a specific pattern was showed; at low dose levels the immobility was lower than modelled (antagonism), whereas at high dose levels the immobility was higher than modelled (synergism). On the other hand, no deviation was observed from independent action in algal tests. This study represents an important step to understand the interactions among pesticides detected previously in field monitoring studies of agricultural areas in “Lezíria do Tejo”, Portugal. Observed mixture toxicity was compared with predictions, calculated from the concentration response functions of chlorpyrifos and terbuthylazine at two realistic concentration ratios by applying the biologically relevant patterns in which deviations occurred. The assumption of these last yielded accurate predictions, although worst for the mixture ratio chlorpyrifos 0.17 and terbuthylazine 85 µg/L under consideration.

Keywords*:* Mixture, chlorpyrifos, terbuthylazine, *Daphnia magna*, *Pseudokirchneriella subcapitata*, toxicity

Resumo Alargado

Os pesticidas são utilizados pelo Homem como forma de aumentar a produtividade agrícola, permitindo a proteção das culturas contra organismos prejudiciais. A procura por estes produtos têm sido influenciada pela globalização, alterações no clima, aumento da população e urbanização, que se têm feito sentir ao longo dos anos.

Apesar das vantagens económicas destes produtos, o seu uso involve riscos e perigos à Saúde Pública e meio Ambiente. Estes riscos ocorrem devido a sua sobreutilização e aplicação incorreta dos mesmos dos mesmos.

Devido à utilização de pesticidas na agricultura, organismos e comunidades presentes naturalmente nos ecossistemas circundantes às culturas, apresentam grande probabilidade de virem a sofrer os efeitos toxicológicos destes produtos (Schäfer *et al.*, 2011). Porém, esta situação pode ser contornada através de uma correta avaliação de risco e gestão de produtos químicos, permitindo fornecer uma base para o uso sustentável de substâncias químicas (Backaus *et al.*, 2010).

Este estudo foi realizado com base num cenário real de exposição, na Lezíria do Tejo, zona centro de Portugal Continental, que reflete a carga média de pesticidas presente na drenagem de campos de áreas agrícolas após tratamentos efetuados aos campos de milho, na primavera. Foi encontrada a combinação do herbicida terbutilazina e do insecticida clorpirifos, nas águas de superfície monitorizadas (Silva *et al.*, 2015, Pereira, *in press*).

Para testar esta mistura realizaram-se bioensaios de espécies individuais, utilizando o microcrustáceo *Daphnia magna* e a microalga *Pseudokirchneriella subcapita*. Foram utilizados Toxkits, em vez de bioensaios convencionais, uma vez que este tipo de testes permite obter uma maior uniformidade das condições de exposição dos organismos bem como reduzir o tempo do teste e o uso de material.

Para prever a toxicidade da mistura, são utilizados dois modelos de referência: Adição de Concentração (AC) (Loewe & Muischnekand, 1926) e Ação Independente (AI) (Bliss, 1939). O modelo AC é geralmente utilizado quando os pesticidas presentes na mistura apresentam modos de ação semelhantes, enquanto que o modelo AI é utilizado para descrever pesticidas com modos de ação diferentes. Estes modelos assumem que não ocorre interação entre as substâncias e, desta forma, é possível prever a toxicidade conjunta de qualquer mistura, utilizando como dados iniciais a toxicidade das substâncias individuais (Cedergreen *et al.*, 2013). Porém, quando se verifica que interações entre as substâncias químicas ocorrem, são encontrados desvios aos modelos de referências, indicados anteriormente. Os tipos de desvios que podem ocorrer são o sinergismo/antagonismo (sendo sinergismo caracterizado por o efeito observado ser mais severo que o efeito

calculado pelo modelo de referência, e o antagonismo por ser menos severo); desvio com dependência ao nível da dose, que indica que os desvios observados são diferentes para as altas e baixas concentrações da gama de concentrações analisada (por exemplo, pode ser observado antagonismo a baixa dose e sinergismo em altas doses de concentração); e o desvio com dependência da proporção da dose; este desvio depende da composição da mistura (por exemplo, numa mistura binária, a substância 1 pode causar o antagonismo observado nos dados enquanto que a substância 2 pode causa sinergismo). Estes desvios foram analisados com auxílio do modelo MIXTOX, desenvolvido por Jonker *et al.* (2005).

Em estudos realizados com misturas binárias é comum utilizar-se superfícies de dose-resposta, de forma a permitir uma melhor visualização das possíveis interações entre os dois químicos, num sistema de teste específico. Estas superfícies descrevem a superfície de concentração-resposta completa, através da conceção de uma estrutura experimental que atribua dados para toda a gama de combinações possíveis entre os dois produtos químicos. O método gráfico geralmente apresentado é o isobolograma, que compara a *isobole* prevista pelo modelo utilizado, com uma combinação de concentrações que causa um efeito predefinido (geralmente utiliza-se o que causa 50% de efeito), extrapolando essas concentrações para um plano x/y (Cedergreen *et al.*, 2013).

Este estudo tem como principais objetivos fornecer maior realismo ambiental à previsão dos riscos associados às misturas de pesticidas bem como às incertezas associadas a situações de exposição agrícola, abordando as seguintes questões: (1) qual a relevância da combinação de efeitos de uma mistura binária de pesticidas (clorpirifos e terbutilazina) encontrada num ambiente aquático, adjacente a um campo de milho em Portugal, para várias concentrações, em espécies selecionadas de dois níveis tróficos diferentes (*D. magna* como consumidor primário e *P. subcapitata* como produtor primário); (2) verificar se os modelos de referência, AC e AI, descrevem bem a toxicidade da mistura a partir da toxicidade das substâncias individuais e, caso não sejam descritas por estes modelos pois apresentam desvios, (3) como é que o modelo MIXTOX caracteriza tais desvios?

Os dados de exposição aos pesticidas testados individualmente permitiram observar que a *D. magna* é sensível a estes pesticidas uma vez que os valores de EC_{50} são caracterizados como muito tóxicos (EC_{50} ≤1 mg/L, EC, 2001). Para a microalga *P. subcapitata*, observou-se para a terbutilazina um valor de EC_{50} muito tóxico, e tóxico para o insecticida clorpirifos (1 ≤ EC_{50} ≤ 10 mg/L, EC, 2001).

Relativamente aos dados de exposição da mistura para a *D. magna*, após ajuste dos dados aos modelos de referência, identificou-se um desvio de dependência da dose ao modelo de referência AI. Este desvio indica que a baixas doses os efeitos foram antagónicos e a altas doses observou-se um sinergismo nos dados. Estes resultados não são coincidentes com estudos anteriores, onde a mistura do clorpirifos com s-triazinas apresenta

geralmente comportamentos sinergéticos (PapeLindstrom & Lydy, 1997; Belden & Lydy, 2000; Anderson & Lydy, 2002; Jin-Clark *et al.*, 2002; Lydy & Linck, 2003; Lydy & Austin, 2004; Banks *et al.*, 2005; Schuler *et al.*, 2005; Trimble & Lydy, 2006; Wacksman *et al.*, 2006; Loureiro *et al.*, 2009; Loureiro *et al.,* 2010; Amorin *et al.*, 2012; Pérez *et al.*, 2013a,b; Yang *et al.*, 2015; Xing *et al.*, 2015). A diferença nos resultados pode ser justificada devido à utilização de espécies e *endpoints* diferentes nesses estudos.

Para os dados relativos à microalga não foram encontrados desvios aos modelos de referência. Assim sendo, o modelo de Ação Independente deveria ser escolhido para caracterizar os dados de toxicidade da mistura, uma vez que os pesticidas apresentam modos de ação diferentes, porém, foi selecionado o modelo de Adição de Concentração, uma vez que fornece previsões mais conservativas, sendo geralmente recomendo para avaliação de risco de pesticidas.

Relativamente ás concentrações observadas em campo, na *D. magna* foram observados efeitos de 45% (mistura 1: clorpirifos a 0,17 µg/L e terbutilazina a 8,5 µg/L) e 75% (mistura 2: clorpirifos a 0,17 µg/L e terbutilazina a 85 µg/L) na sua mobilidade. Os resultados para a mistura 1 vão de encontro à previsão obtida com o modelo de Ação Independente com desvio dependente do nível da dose, enquanto na mistura 2 os resultados são mais elevados que os previstos no padrão de desvio. Os efeitos observados na inibição de crescimento da *P. subcapitata* foram de 31% e 88% para a mistura 1 e 2, respetivamente, apresentando concordância com os valores previstos pelos modelos de referência.

Estes estudo revela uma diferença na sensibilidade das espécies à exposição dos pesticidas e alerta para a falta de informação dos testes de toxicidade realizados para a exposição individual de pesticidas.

Como conclusão final, o modelo MIXTOX, descrito em Jonker *et al.* (2005), permitiu fazer uma boa caracterização da toxicidade da mistura e dos desvios que ocorrem aos modelos de referência. Assim sendo a utilização de misturas realistas pode permitir uma melhor avaliação de risco de pesticidas, uma vez que a partir deste tipo de dados pode-se descrever uma probabilidade de falha dos modelos de referência e analisar a existência de uma padrão de desvios. Isto poderá permitira introdução de uma factor de segurança para a toxicidade de misturas bem como uma melhor compreensão dos efeitos mecanisticos das mesmas.

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List of Abbreviations

Abbreviation	Designation
AChE	Acetylcholinesterase
ANIPLA	“Associação Nacional da Indústria para a Proteção das Plantas”
AZ	Azoles
CA	Concentration Addition
CAS	Chemical Abstracts Service
ChE	Cholinesterase inhibitors
conc	Concentration
CPF	Chlorpyrifos
DL	Dose level-dependent
DR	Dose ratio-dependent
EC	Effect Concentration
EFSA	European Food Safety Authority
EU	European Union
EPA	Environmental Protection Agency
h	Hours
IA	Independent Action
INE	Instituto Nacional de Estatística
ISO	International Organization for Standardization
m	Million
MCPA	2-methyl-4-chlorophenoxyacetic
MOA	Mode of Action
MDR	Model Deviation Ratio
NA	Quantity is not applicable
OECD	Organization for Economic Co-operation and Development
PPP	Plant Protection Products
PPR	Plant Protection Products and their Residues
r^2	Coefficient of regression
S/A	Synergism/Antagonism

SOP	Standard Operating Procedure
SS	Sum of Squares
TBZ	Terbuthylazine
TU	Toxic Unit
TZ	Triazines
UAA	Utilised Agricultural Area

1. Introduction

Plant production has a very important place in the Community (EC, 2009). One of the most important ways of protecting plants and plant products against harmful organisms, including weeds, and of improving agricultural production is the use of plant protection products (PPP).

However, PPP can also have non-beneficial effects on plant production. Their use may involve risks and hazards for humans, animals and the environment, especially if placed on the market without having been officially tested and authorised and if incorrectly used. Since the use of pesticides in agriculture inevitably leads to exposure of non-target organisms (including humans), undesirable side-effects may occur on some species, communities or on ecosystems as a whole (van der Werf, 1996). Given the large amounts of pesticides applied globally and given the fact that they are designed to harm biota, there is a high potential for adverse environmental effects also on non-target communities (Schäfer *et al.*, 2011)

A correct risk assessment and management of chemicals is the basis for any chemical control and risk reduction measures and ultimately provides a basis for the sustainable use of substances (Backhaus *et al.*, 2010).

Since there is a multitude of chemicals in all environmental compartments and in exposed biota, it is impossible to test each and every imaginable mixture. These mixtures found in the environmental can be analysed using two different approaches: “top-down” and the “bottom-up”. The “top-down” approaches has based on complex mixtures extracted from biological tissue or environmental samples, trying to identify the individual compounds that contribute to the observed toxicity of the samples. On the other hand, “bottom-up” approaches predict the toxicity of a defined mixture, based on a priori knowledge of the chemical composition and toxicity of the mixture components (Pérez *et al.*, 2011).

This study is based on a site-specific exposure scenario that reflects the median load of pesticides in field drainage in Central-Portuguese agricultural areas after maize treatments in spring. In small agricultural streams (and other edge-of-field surface waters) the herbicide terbuthylazine and the insecticide chlorpyrifos are strongly dominating the toxicity at the same time and/or in sequence. This has been shown in field monitoring studies of agricultural areas in “Lezíria do Tejo”, Portugal (Silva *et al.*, 2015, Pereira, *in press*). Considering this, a “bottom-up” approach was chosen to assessed the derive patterns for toxicity response of the mixture.

In order to predict the mixture toxicity, two concepts were used, usually termed Concentration Addition (CA) (Loewe & Muischnekand, 1926) and Independent Action (IA) (Bliss, 1939); allow predicting the joint toxicity of chemicals in any mixture, using the single-

substance toxicity information as input data and assuming non-interaction (Cedergreen *et al.*, 2013).

It has performed various investigations in order to analyse the power of both concepts in typical toxicological and ecotoxicological assays and for a range of different environmental chemicals. Several studies suggest that mixtures of contaminants that have the same mode of action (MOA) have a better fit to the CA model and the mixtures with different MOA tend to be best modeled by IA (Faust *et al.*, 2000; Altenburger *et al.*, 2003; Backhaus *et al.*, 2004b). However, can occur deviations from these reference models. There are three types of deviation that can occur: synergism/antagonism (S/A), dose level dependency (DL) and dose ratio dependency (DR). To model these deviations, it was used the MIXTOX model, proposed by Jonker *et al.* (2005). The size of the deviation from a reference model can also be analysed using indices and graphical methods. The called model deviation ratio (MDR) is usually used; this presents the ratio between the predicted effect concentration and observed effect concentration (Belden *et al.*, 2007).

In studies with binary mixtures, the dose-response surfaces are common used to visualise the possible interactions between two chemicals in a specific test system, but also the most elaborate, data-demanding approach. These dose-response surfaces describe the entire concentration-response surface by designing an experiment that administer data for the full range of possible combinations between two chemicals. The graphical method normally used is the isobologram, where is possible to compare the predicted isobole to a concentration combination that cause a predefined effect (usually 50%) from each ratio, extrapolating these concentrations to the x/y plane (Cedergreen *et al.*, 2013).

The usual organization for this type of study involve 1) the determination of the toxicity of the mixtures components; 2) the use of these data to predict the mixture toxicity according to CA and/or IA models; and finally 3) the comparison of the experimentally observed mixture toxicity with the conceptual predictions (Cedergreen *et al.*, 2013).

These analyses will be done with well-known and established single-species assays using the green limnic microalgae *Pseudokirchneriella subcapita* and the microcrustacean *Daphnia magna*.

Few studies tested the effect of binary mixtures consisted of terbuthylazine and chlorpyrifos. Munkegaard *et al.* (2008) investigated whether interactions between the two pesticides can take place in the aquatic algae *P. subcapitata* and the aquatic macrophyte *Lemna minor*. Changes in swimming behaviour and the inhibition of AChE were related and synergistic patterns were observed when *Danio rerio* (Pérez *et al.*, 2013a) and *Chironomus riparius* larvae (Pérez *et al.*, 2013b) were exposed to chlorpyrifos mixtures containing atrazine and terbuthylazine. Apart of this, no mixture study with terbuthylazine or any *s*-

triazine has, to our knowledge been done, in *D. magna*, using a ray design and response surface analyses.

Most studies are restricted specifically to “reference mixtures”, in which all the components were known to act either by an identical or by completely different molecular mechanisms of action (Backhaus & Faust, 2012; Junghans *et al.*, 2006). The experimental investigations on the predictive value of the concepts with environmentally realistic mixtures have been rarely conducted, hence, our knowledge on their performance under these circumstances is limited.

This study aims to overcome this limitation by comparatively analysing the predictive value of both concepts, CA and IA, for an example of a typical environmentally realistic mixture of pesticides.

In conclusion, the specific objectives of this study were to achieve more environmental realism in the scientific basis for forecasting risks and associated uncertainties of agricultural exposure situations by addressing the following questions:

- how relevant are combination effects of a binary mixture of pesticides (chlorpyrifos and terbuthylazine) that was found (or that co-occurred) in the aquatic environment under a maize field condition within Portugal, at different measured concentrations, on selected species (*P. subcapitata* and *D. magna*) of two different trophic levels (primary producers and primary consumers), and
- may the toxicity of such pesticide mixture be predictable from single substances toxicity data using the descriptive models, CA and IA, and if not, how the deviations from them are characterised using the MIXTOX model?

2. State of the art on the evaluation of the aquatic risk of pesticide mixtures

2.1. Importance of plant protection products in Agriculture

Agriculture has a very important role in the European Union (EU) economy. Latest figures show that farming employs over 20 million people in the 28 EU Member States, many of which are in peripheral regions and rural. The Utilised Agricultural Area (UAA) in the EU-28 it is from 174351010 hectares and in Portugal are about 3 641 600 hectares, with 264400 holdings. In the EU-28, 59.8 % of the UAA was used as arable land. In the Mediterranean countries, like Cyprus, Greece and Portugal, the proportion of UAA occupied by permanent crops was relatively high (a little over 19 %) (Forti & Hemrard, 2016).

One of the most important ways of protecting plants and plant products against harmful organisms, including weeds, and of improving agricultural production is the use of plant protection products (PPP), generally known as pesticides. The use of PPP is associated with global impacts felt on agriculture, result of globalization, climate change, population increase and urbanization.

In 2012 and 2013, it was recorded in Portugal about 1.084 million hectares of crops potentially treated with PPP, and 58% of this surface was effectively treated. It should be noted that herbicides and fungicides account for 39% and 37% of hectares treated in Portugal, respectively, followed by the areas treated with insecticide, which represent 26% of the total area treated with pesticide. In those same years, the pesticides with the greatest application were glyphosate, terbuthylazina and s-metolachor, respectively. In addition, the most applied insecticides were paraffin oil, chorpyrifos and dimethoate, respectively (Figure 1. INE, 2015).

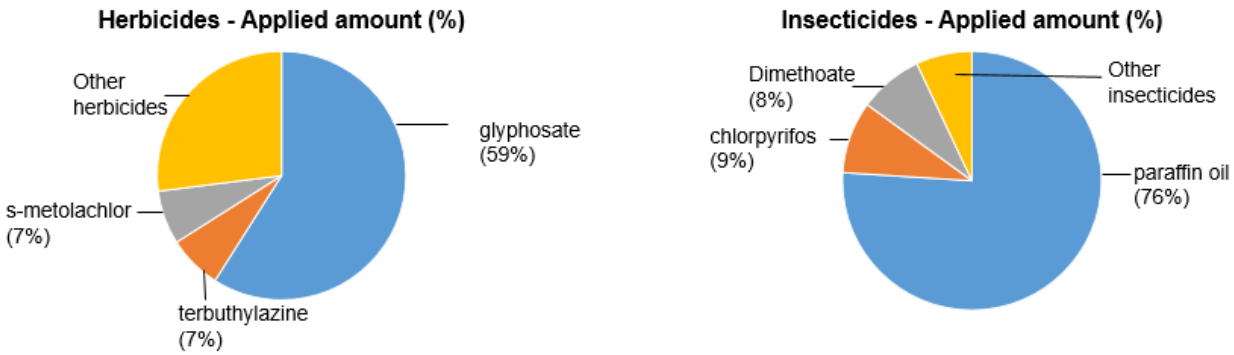

Figure 1 (Chapter 2). Percentage of herbicides and isecticides applied in Portugal, in 2012 and 2013 (Adapted from INE, 2015).

After some stagnation in the recent past the plant protection products sector in Portugal rise 7.5% in 2014, setting up above the 118 million euros, more 8 m€ that in 2013. The increase was driven by the occurring climatic conditions that favored the incidence of diseases in crops, leading to increased application of fungicidal. Due to rainfall levels recorded this year, there was an increase in the development of weeds, leading to increased application of herbicides (Anipla, 2015). The analysis of sales is very important since it may be indirectly related to the amount used thereof, which may vary depending on weather conditions or disease problems.

In 2014, sales of PPP were the dominated by fungicides (64% of total sales volume), followed by herbicides (18.7%) and insecticides and acaricide (5.7%) (INE, 2016).

The pesticides used in this study was terbuthylazine (Chemical Abstracts Service [CAS] no. 5915-41-3) and chlorpyrifos (CAS no. 2921-88-2). Terbuthylazine herbicide belongs to the family of s-triazines, and it was first used as a substitute of atrazine essentially in corn and tomato crop. Compared with other triazine pesticides, terbuthylazine is a relevant greater persistence (Navarro *et al.*, 2004). This herbicide causes inhibition of photosynthesis at photosystem II receptor site (HRAC, 2016).

The insecticide chlorpyrifos belongs to the organophosphate class, which acts as an inhibitor of acetylcholinesterase (AChE) and, consequently, the transmission of nerve impulses across the synaptic junctions between nerve (Loureiro *et al.*, 2010). According to EC (2013), chlorpyrifos is described as a priority hazardous substance in the field of water policy. This insecticide is recommended against a large number of pests in different crops (INE, 2015).

2.2. Uncertainties in the assessment of the aquatic risk of pesticides

Plant protection products can have non-beneficial effects on plant production. Their use may involve risks and hazards for humans, animals and the environment, especially if placed on the market without having been officially tested and authorised and if incorrectly used. Since the use of pesticides in agriculture inevitably leads to exposure of non-target organisms (including humans), undesirable side-effects may occur on some species, communities or on ecosystems as a whole (van der Werf, 1996). Given the large amounts of pesticides applied globally and given the fact that they are designed to harm biota, there is a high potential for adverse environmental effects also on non-target communities (Schäfer *et al.*, 2011). Unacceptable effects of pesticides in the field were identified in several studies, examples include investigations by Dabrowski *et al.* (2001); EFSA PPR (2013); Liess & Schulz (1999); Liess and von der Ohe (2005); Liess *et al.* (2005); Muschal & Warne (2003); Schäfer *et al.* (2012). One of the possible reasons for the effects observed could be the

failure of risk assessment to have predicted/precluded these effects, since it traditionally focuses on single stressors. Examples that certainly will underestimate the risk are tank mixtures, while multiple exposure (serial application of several PPP, multi-crop, multi-year sequential pesticide exposure) effects of pesticides, long-term delayed effects of pesticides and combined effects between the PPP and environmental stressors as hydrodynamic stress (EFSA PPR Panel, 2013).

2.3. Prediction models for mixtures of pesticides toxicity

Since there is a multitude of chemicals in all environmental compartments and in exposed biota, it is impossible to test each and every imaginable mixture. However, the two concepts, usually termed Concentration Addition (CA) (Loewe & Muischnekand, 1926) and Independent Action (IA) (Bliss, 1939), allow predicting the joint toxicity of chemicals in any mixture, using the single-substance toxicity information as input data and assuming non-interaction (Cedergreen *et al.*, 2013).

The CA model is defined as a summation of relative toxicities of the individual toxicants in the mixture (Loewe & Muischnekand, 1926); its mathematical expression is as follows:

$$\sum_{i=1}^{n} \frac{C_i}{EC_{xi}} = 1 \quad (1)$$

Where C_i are the concentrations of the individual components in the mixture and EC_{xi} are the equivalent effect concentrations of the single substances, i.e. concentrations that alone would cause the same quantitative effect x as the mixture. These quotients express the toxic unit (TU), which represents the concentrations of mixture components as fractions of equi-effective individual concentrations.

On the other hand, the IA model is based on the idea of a dissimilar action of the mixture components (Bliss, 1939), generating independence toxicities probabilities of mixture components (Altenburger *et al.*, 2004; Jonker *et al.*, 2005). The mathematical expression is as follows:

$$Y = u_{max} \prod_{i=1}^{n} q_i\,(C_i) \quad (2)$$

Where Y is the biological response, C_i the concentration of the component i in the mixture, u_{max} the control response for endpoints and $q_i(C_i)$ the probability of non-response.

The challenge is to test how well these concepts describe the mixture toxicity in actual biological systems of varying complexity. Two main approaches can be noted: The “top-down” and the “bottom-up” approach. The top-down approaches has based on complex

mixtures extracted from biological tissue or environmental samples, trying to identify the individual compounds that contribute to the observed toxicity of the samples. The CA and IA models are employed in as tools for connecting toxicities of the complex mixtures to that of the individual compounds and to identify knowledge gaps (Brack, 2003; Brack *et al.*, 2007, 2008, 2015, 2016; Burgess *et al.*, 2013; Cedergreen *et al.*, 2013; Grote *et al.*, 2005).

Bottom-up approaches predict the toxicity of a defined mixture, based on a priori knowledge of the chemical composition and toxicity of the mixture components. The aims of this approach is to test the predictive power of CA and IA for certain chemicals and biological test system, analyse deviations from conceptual expectations (interactions), and provide quality targets for chemical mixtures (Vighi *et al.*, 2003). In both approaches, the application of CA and/or IA can be hampered by interactions between the mixture components (Cedergreen *et al.*, 2013).

It has performed various investigations in order to analyse the power of both concepts in typical toxicological and ecotoxicological assays and for a range of different environmental chemicals. The usual organization for this type of study involve 1) the determination of the toxicity of the mixtures components; 2) the use of these data to predict the mixture toxicity according to CA and/or IA models; and finally 3) the comparison of the experimentally observed mixture toxicity with the conceptual predictions (Cedergreen *et al.*, 2013).

Several studies suggest that mixtures of contaminants that have the same mode of action (MOA) have a better fit to the CA model and the mixtures with different MOA tend to be best modeled by IA (Faust *et al.*, 2000; Altenburger *et al.*, 2003; Backhaus *et al.*, 2004b). In mixtures with the same MOA, the CA predict accurately the toxicity and IA under predicted toxicity. However, model selection may be governed more appropriately by the goal of selecting the more conservative of the models that is the CA model, this model tends to provide more conservative estimates of toxicity compared to the IA model, yet with quite similar overall predictive accuracy. Drescher and Boedeker (1995) have previously theorized that the conservative nature of the model CA occurs when dose-response slopes are steep, recurring situation for most aquatic pesticide exposures (Belden *et al.*, 2007).

The most informative approach to investigate the joint toxicity of a binary mixture is to create complete concentration-response surfaces, although this method is very data-demanding (Cedergreen *et al.*, 2013). Greco *et al.* (1995) reviewed several methods to create and evaluate concentration-response surfaces. Some studies provide statistical tools for concentration-response surface evaluations (Jonker *et al.*, 2005; Sørensen *et al.*, 2007; White *et al.*, 2004).

Concentration-response surfaces were mainly used to view graphically the possible interactions between two chemicals in a specific test system. These surfaces can be a

helpful tool to understanding terms such as mixture ratios, dose levels, and isobolograms, which are so often used in mixture toxicity studies (Cedergreen *et al.*, 2013).

The use of response surfaces allows to describe the entire concentration-response surface by designing an experiment that administer data for the full range of possible combinations between two chemicals (Cedergreen *et al.*, 2013). Usually this is done using a full ray design or a factorial design where fixed chemical concentrations are combined (Greco *et al.*, 1995). These surfaces are the most robust statistical method that evaluate whether data can be described with CA and/or IA (Cedergreen *et al.*, 2013).

After design and implementation of the experiment, the results can be compared with the predictions given by CA and/or IA using the concentration-response data from the single exposures. This comparison can be performing graphically or statistically (Cedergreen *et al.*, 2013). The best practice is to perform both, in order to obtain a fullest picture of how well the experimental data are predicted by IA and/or CA (Jonker *et al.*, 2005). One graphical comparison method widely used is the isobole concept, where data and model predictions at chosen effect levels are tested separately. In isobolograms is possible to compare the predicted isobole to a concentration combination that cause a predefined effect (usually 50%) from each ratio, extrapolating these concentrations to the x/y plane (Cedergreen *et al.*, 2013).

To evaluate the adjustment of the data to the reference models (CA or IA), data needs to be describes with an extended reference model that includes parameters that describe relevant deviations from the previous simpler model. If the data is arrange so the two models are mathematically nested, the fits can be compared statistically using likelihood ratio statistics. If the data is not described in greater significance for the more complex model than the simple reference model, then it means that the data do not show deviations from the reference models (Jonker *et al.*, 2005; Sørensen *et al.*, 2007).

2.4. Deviations from the reference models

There are different indices and graphical methods that can be used to quantify the size of the deviation from a reference model (Altenburger *et al.*, 2003). One of these indices is called model deviation ratio (MDR), it is the ratio between the predicted effect concentration (usually the EC_{50} or LC_{50}) and observed effect concentration. This ratio allows identifying synergism (MDR > 2), additivity ($0.5 \leq MDR \leq 2$) and antagonism (MDR < 0.5) (Belden *et al.*, 2007).

The chemical mixture and mixed stressor experiments were mainly undertaken and analysed using the experimental design and data analysis framework developed within NoMiracle (Løkke, 2010). Use of this framework (the MIXTOX model) allowed modelling of

response surfaces using an optimal design and minimum number of test animals (Jonker *et al.*, 2005). Starting from effect prediction of CA and IA, effect data-sets are analysed to see how observed combined toxic effects compared with expected effects as calculated using the CA or IA models. Analysing if and how the observed data deviates from the reference CA or IA model predictions, is possible to enable characterization of the data set with respect to interactions that cause actual effects and that may cause deviations. Four types of joint effect are considered to have the most biologically significance:

1. No deviation from Concentration Addition or Independent Action (CA or IA);

2. Synergism or Antagonism (S/A): The effect observed is more severe (synergism) or less severe (antagonism) than the effect calculated with either reference models;

3. Dose Level dependent deviation (DL): The deviation from the reference models is different at low and high dose levels. For example, antagonism may be observed at low dose levels and synergism at high;

4. Dose Ratio dependent deviation (DR): The deviation depends on the composition of the mixture. In a binary mixture, the toxicant 1 may be the cause of the observed antagonism, whereas the toxicant 2 can cause the effect of synergism.

For the synergy/antagonism deviation model (S/A model), the extra parameter *a* can become negative or positive, respectively, for both reference models. When a=0, the S/A model reduces to the CA or IA. A second parameter b_{DL} con be included in addition to *a*, in order to generate the dose-level (DL) deviation model. In this case the value of *a* indicates the deviation at low doses (i.e., a>0=antagonism, and a<0=synergism) and the value of b_{DL} indicates at what dose level the deviation changes (i.e., from synergism to antagonism or vice versa). For CA/DL, the dose level where the deviation change occurs can be calculated using the follow expression: $1/b_{DL} \cdot EC_{50}$; e.g., $b_{DL}=1$ means that the switch occurs at the EC_{50} isobole. When $b_{DL}=0$, the equation reduces to the S/A model. If $b_{DL}<0$, the magnitude of synergism/antagonism (*a*) becomes dose-level dependent, but does not switch. In IA/DL deviation function, the switching can be estimated directly from $1/b_{DL}$; the switching occurs at mixture doses that cause a specific level of effect. If $b_{DL}=2$, the switching occurs at doses where effect level is 50%. If $b_{DL}=0$, the deviation function again reduces to the S/A model. When $b_{DL}<1$, the magnitude of synergism/antagonism becomes response-dependent, but does not switch (Loureiro *et al.*, 2010).

For dose-ratio (DR)-dependency, again a second parameter b_{DR} is included in addition to *a*. The extra parameter b_{DR} express the dependency of the reference models on the composition of the mixture. In a binary mixture, antagonism can be observed where the toxicity of the mixture is caused mainly by toxicant 1, whereas synergism can be observed

where the toxicity is mainly caused by toxicant 2. Therefore, the b_{DR} relates to the lead chemical of the mixture (i.e., the one mentioned and modelled first). In DR model, the parameter *a* quantifies the degree of antagonism (a>0) or synergism (a<0) and a significant b_{DR} quantifies the degree of reduced (b_{DR}>0) or increased (b_{DR}<0) toxicity due to the lead chemical. When *a* and b_{DR} have opposing signs, occurs a switch between antagonism and synergism within the response surface; whereas, if they have the same sign, the magnitude of the antagonism or synergism will vary with the ratio of chemicals, but not switch (Loureiro *et al.*, 2010).

The MIXTOX model allows the analysis of deviations from the reference models. In application of the model are introduced extra parameters whose values indicate the type of deviation that occurs. For more details, checked on Table 1 and also in the work described by Jonker *et al.* (2005).

Table 1 (Chapter 2). Interpretation of additional parameters (a and b) that define the functional form of deviation pattern from concentration addition (CA) and independent action (IA) (Adapted from Jonker *et al.*, 2005).

Deviation Pattern	Parameter *a* (CA or IA)	Parameter *b* (CA)	Parameter *b* (IA)
S/A	$a > 0$: Antagonism	-	-
	$a < 0$: Synergism	-	-
DL	$a > 0$: Antagonism at low dose level and synergism at high dose level	$b_{DL} > 1$: Change at lower EC_{50} level;	$b_{DL} > 2$: Change at lower EC_{50} level;
		$b_{DL} = 1$: Change at EC_{50} level	$b_{DL} = 2$: Change at EC_{50} level
	$a < 0$: Synergism at low dose level and antagonism at high dose level	$0 < b_{DL} < 1$: Change at higher EC_{50} level;	$0 < b_{DL} < 2$: Change at higher EC_{50} level;
		$b_{DL} < 1$: No change but the magnitude of S/A is DL dependent	$b_{DL} < 1$: No change but the magnitude of S/A is effect level dependent
DR	$a > 0$: Antagonism, except for those mixtures ratios where significant negative b_is indicate synergism	$b_i > 0$: Antagonism where the toxicity of the mixture is caused mainly by toxicant i	
	$a < 0$: Synergism, except for those mixtures ratios where significant positive b_is indicate antagonism	$b_i < 0$: Synergism where toxicity of the mixture is caused mainly by toxicant i	

2.5. Studies of quantification of synergism

There are several studies which demonstrate synergism (MDR > 2) in pesticides mixtures, which is the case of Belden *et al.* (2007) and the review by Cedergreen *et al.* (2008), in addiction there are another 84 papers reviewed in Cedergreen (2014). This review resulted in a database on synergistic interactions including 73 cases of synergy from both Belden *et al.* (2007) and the data search compiled from 36 studies. The effects of 54 pesticides combinations were tested on 27 different species. Of all the combinations tested, 69 were binary mixtures while the remaining four mixtures consisted of combinations of three or five organophosphate insecticides or eight chloroacetamide herbicide safeners. If pesticides are divided into groups that have the same MOA, Cedergreen (2014) showed that particularly five groups of pesticides were overrepresented in the synergistic mixtures: organophosphate and carbamate insecticides (Cholinesterase inhibitors), azole fungicides (Ergosterol biosynthesis inhibitors), triazine herbicides (Photosystem II inhibitors) and pyrethroid insecticides (interferes with sodium channels in nerve cells) (Figure 2A). Looking which of the binary mixtures of the above pesticide groups that induce synergism deviation in auto-trophic organisms (plants and algae) and hetero-trophic organisms (microorganisms and animals), and arrange the cholinesterase inhibitors together, it is possible to observe that there are no cases of synergy within the autotrophic organisms (Figure 2B). For the hetero-trophic organisms, 69 of the 73 synergistic mixtures (95%) contained either cholinesterase inhibitors (organophosphates or carbamates) or azole fungicides (Figure 2C). The remaining mixtures are the mixtures of the 8 herbicide safeners, pyrethroid with an organochloride insecticide, a pyrethroid insecticide and a piperidine fungicide and a photosystem II (PSII). Of the 69 binary mixtures, 24% contained an azole fungicide and 76% contained a cholinesterase inhibitor (Figure 2C). The group of triazines only entered in synergistic mixtures in combination with either chlorpyrifos, diazinon, malathion, methidathion, methyl-parathion, which belong to the phosphorothioate and phosphorodithioates class of organophosphates, or trichlorfon, a phosphate class organophosphate. On the other hand, pyrethroids only entered in synergistic mixtures where combined with azole fungicides (Cedergreen, 2014).

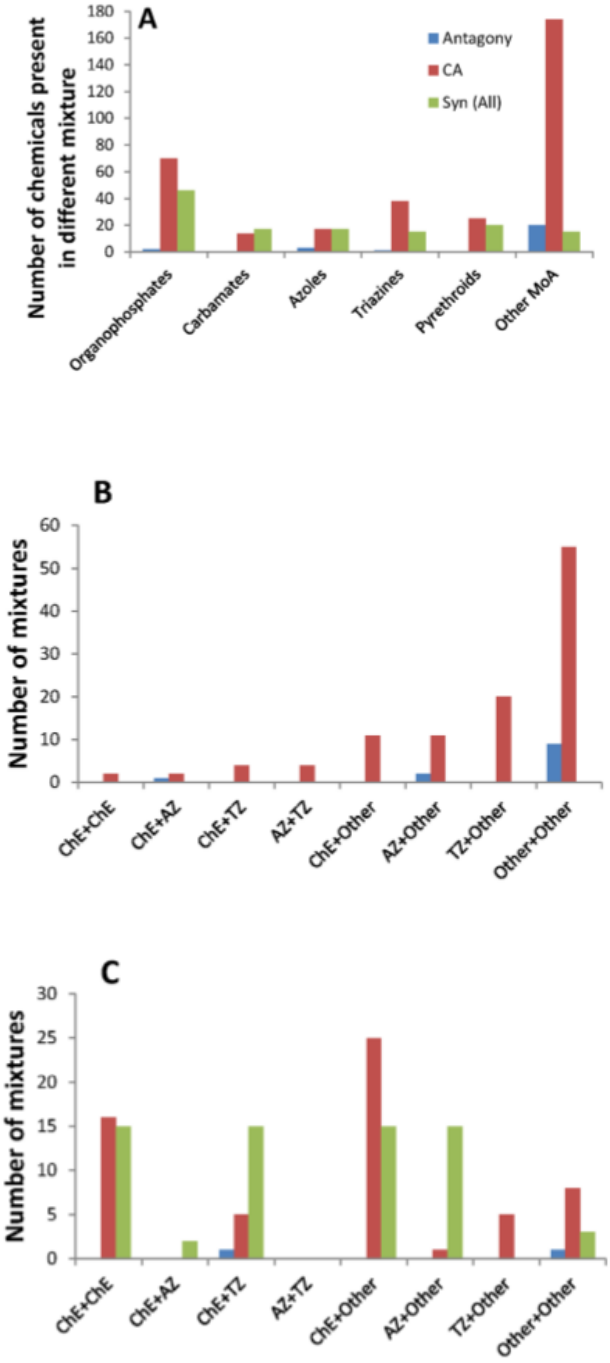

Figure 2 (Chapter 2). Frequency of pesticide antagony, additivity and synergy. Figure 2A shows the number of times a pesticide belonging to the group organophosphates, carbamates, azoles, triazines, pyrethroids or some other Mode of Action (other MoA) occur in a binary mixture resulting in antagony (blue bars), concentration additivity (CA) (red bars) or synergy (green bars). In figure 2B and 2C, the number of binary combinations of cholinesterase inhibitors (ChE), azoles (AZ), triazines (TZ) and other Modes of Action (Other) resulting in either antagony, concentration additivity or synergy are shown for mixtures tested on 2B autotropic organisms (plants and algae, n = 120) or 2C for heterotrophic organisms (microorganisms and animals, n = 128) (Adapted from Cedergreen, 2014).

2.6. Importance of evaluating the aquatic toxicity of realistic mixtures

The comparative evaluation of the predictive potential of CA and IA, however, has so far been largely restricted to such specifically designed “reference mixtures”, in which all the components were known to act either by an identical or by completely different molecular mechanisms of action (Backhaus & Faust, 2012; Junghans *et al.*, 2006). This situation might be considered atypical for actual mixtures found in the environment. As experimental investigations on the predictive value of the concepts with environmentally realistic mixtures have been rarely conducted, our knowledge on their performance under these circumstances is limited. This study aims to overcome this limitation by comparatively analysing the predictive value of both concepts, CA and IA, for an example of a typical environmentally realistic mixture of pesticides. It is based on a site-specific exposure scenario that reflects the median load of pesticides in field drainage in Central-Portuguese agricultural areas after maize treatments in spring. In small agricultural streams (and other edge-of-field surface waters) the herbicide terbuthylazine and the insecticide chlorpyrifos are strongly dominating the toxicity at the same time and/or in sequence. This has been shown in field monitoring studies of agricultural areas in “Lezíria do Tejo”, Portugal, where some of the most important irrigated crops are maize, tomato for industry, rice, sugar beet, potato and open-air horticultural (Silva *et al.*, 2015; Pereira, *in press*).

Other authors have present exposure studies were the mixture with these two pesticides was found (research conducted on the “*Web of Science*” with the keywords: “pesticides”, “exposure”,”water”, “chlorpyrifos”, “terbuthylazine”), these are listed in the table below (Table 2).

Table 2 (Chapter 2). Exposure studies were the combination of terbuthylazine and chlorpyrifos were detected.

Authors	Year	Country	Location monitored	Pesticides detected
Palma *et al.*	2010	Portugal	Alqueva Reservoir	atrazine, chlorpyrifos, endosulfan sulphate, simazine and terbuthylazine
Campillo *et al.*	2013	Spain	Mar Menor	atraton, atrazine, chlorpyrifos, chlorpyrifos-methyl, chlortal, prometryn, prometon, pendimethalin, propazine, propyzamine, simazine terbuthylazine-desethyl, terbuthylazine and tributhylphosphate
López-Roldán *et al.*	2013	Spain	Llobregat River	chlorpyrifos, diazinon, terbuthylazine, MCPA and lindane
Moreno-Gonzaléz *et al.*	2013	Spain	Mar Menor	chlorpyrifos, chlortal-dimethyl, diazinon, fluoranthene, fluorine, flutolanil, naphthalene, myclobutanil, phenanthrene, propazine, propyzamide, terbuthylazine, terbutryn, simazine and terbumeton
Silva *et al.*	2015	Portugal	Mondego, Sado and Tejo Rivers	alachlor, atrazine, chlorfenvinphos, chlorpyrifos, endosulfan, ethofumesate, MCPA, metolachlor, metribuzin, molinate, oxadiazon, pendimethalin, profoxydim, propanil, simazine, terbuthylazine, terbutryn, triclopyr
Tsaboula *et al.*	2016	Greece	Pinios River Basin	acetochlor, alachlor, chlorpyrifos, chlorthal, dimethyl, diphenylamine, etridiazole fluometuron, lindane, prometryn, S- metolachlor, terbuthylazine and imidacloprid

The task of the model fitting is done through a suite of modelling tools (Jonker *et al.*, 2005). These analyses will be done with well-known and established single-species assays using the green limnic microalgae *Pseudokirchneriella subcapita* and the microcrustacean *Daphnia magna*.

Taking into account the “Online Database on the Toxicity of Chemical Mixtures” (MixToxDB^{TM}, 2014), and CREST (2016), several studies provide known toxicities caused by combined effects (e.g. additivity, synergism, antagonism, and potentiation) of binary mixtures

using s-triazines and chlorpyrifos in multiple taxa. Species used for testing included auto-trophic organisms: *Dunaliella tertiolecta*, and hetero-trophic organisms: *Chironomus tentans*, *Chironomus riparius*, *Cyprinus carpio* L., *Danio rerio*, *Eisenia fetida*, *Hyalella azteca*, *Lepomis macrochirus*, *Pimephales promelas* (<24h old and 80 days), *Rana clamitans* and *Xenopus laevis* (stage 45 and 35). Table 3 presents the studies were the organisms referred above were used.

Table 3 (Chapter 2). Studies using binary mixtures of s-triazines and organophosphate insecticides.

Authors	Year	Organisms	Class	Conclusions
PapeLindstrom & Lydy	1997	*Chironomus tentans*	Insecta	Synergism between atrazine and various organophosphorous insecticides
Belden & Lydy	2000	*Chironomus tentans*	Insecta	Synergism in the mixture of atrazine with chlorpyrifos, methyl parathion and diazinon
Anderson & Lydy	2002	*Hyalella azteca*	*Crustacea*	Atrazine increased the toxicity of chlorpyrifos
Jin-Clark *et al.*	2002	*Chironomus tentans*	Insecta	Synergism in the two triazine herbicides mixtures with chlorpyrifos
DeLorenzo & Serrano	2003	*Dunaliella tertiolecta*	*Chlorophyta*	Additivity for atrazine and chlorpyrifos mixture
Lydy & Linck	2003	*Eisenia fetida*	*Annelida*	Atrazine and cyanazine increased the toxicity of chlorpyrifos
Lydy & Austin	2004	*Chironomus tentans*	*Insecta*	Additivity for most of the binary mixtures
Banks *et al.*	2005	*Ceriodaphnia dubia*	*Crustacea*	Greater then additivity at environmental relevant concentration of atrazine and diazinon
Schuler *et al.*	2005	*Chironomus tentans*	*Insecta*	Potentiation

Table 4 (Chapter 2). Studies using binary mixtures of s-triazines and organophosphate insecticides.

Authors	Year	Organisms	Class	Conclusions
Trimble & Lydy	2006	*Hyalella azteca*	*Crustacea*	Synergism
Wacksman *et al.*	2006	*Lepomis macrochirus*	*Osteichthyes*	No effect of atrazine on chlorpyrifos toxicity was observed
		Pimephales promelas	*Osteichthyes*	Addition of atrazine increased significantly the chlorpyrifos toxicity
		Rana clamitans	*Amphibia*	No effect of atrazine on chlorpyrifos toxicity was observed
		Xenopus laevis	*Amphibia*	The presence of atrazine increase the toxicity of chlorpyrifos
Munkegaard *et al.*	2008	*Lemna minor*	*Liliopsida*	No deviation from the reference models
		Pseudokirchneriella subcapitata	Algae	
Loureiro *et al.*	2009	*Enchytraeus albidus*	*Enchytraeidae*	Antagonism patterns in mixtures of dimethoate and atrazine, and synergism for lindane and dimethoate

Table 5 (Chapter 2). Studies using binary mixtures of s-triazines and organophosphate insecticides.

Authors	Year	Organisms	Class	Conclusions
Loureiro *et al.*	2009	*Porcellionides pruinosus*	*Isopoda*	-
Amorim *et al.*	2012	*Folsomia candida*	*Collembola*	Synergistic Patterns
Pérez *et al.*	2013a	*Danio rerio*	*Osteichthyes*	Synergism
Pérez *et al.*	2013b	*Chironomus riparius*	*Insecta*	Potentiation
Yang *et al.*	2015	*Eisenia fetida*	*Annelida*	Antagonism in artificial soil
Xing *et al.*	2015	*Cyprinus carpio* L.	*Chordata*	Atrazine and chlorpyrifos induces the occurrence of oxidative stress

Few studies tested the effect of binary mixtures consisted of terbuthylazine and chlorpyrifos. Munkegaard *et al.* (2008) investigated whether interactions between the two pesticides can take place in the aquatic algae *P. subcapitata* and the aquatic macrophyte *Lemna minor*. Changes in swimming behaviour and the inhibition of AChE were related and synergistic patterns were observed when *Danio rerio* (Pérez *et al.*, 2013a) and *Chironomus riparius* larvae (Pérez *et al.*, 2013b) were exposed to chlorpyrifos mixtures containing atrazine and terbuthylazine. Apart of this, no mixture study with terbuthylazine or any *s*-triazine has, to our knowledge been done, in *D. magna*, using a ray design and response surface analyses.

3. Materials and Methods. Results, Discussion and Conclusions

The materials and methods as the study results and their discussion are presented on the following pages in article format to be later submitted to a journal.

Toxicity assessment and prediction of a realistic pesticide mixture from a Portuguese agricultural area using concentration-response surface statistics

3.1. Abstract

Previous work showed the co-occurrence of the organophosphate chlorpyrifos and the s-triazine herbicide terbuthylazine in surface waters of agricultural areas in “Lezíria do Tejo”, Portugal. In the present study, we examine the effects of these pesticides singly and as a binary mixture on the immobility of *Daphnia magna* and on the growth rate of the microalgae *Pseudokirchneriella subcapitata*. Terbuthylazine and chlorpyrifos at single exposure caused a very toxic or toxic response in both organisms. Usually, the toxicity of mixtures is evaluated in relation to the reference models Concentration Addition (CA) and Independent Action (IA). Initially, in this study was used the CA and IA model was to evaluate the joint effects of chlorpyrifos and terbuthylazine. For immobility endpoint, the data fits better to the IA model, due to different mode of action of the pesticides, however a specific pattern was showed; at low dose levels, the immobility was lower than modelled (antagonism), whereas at high dose levels the immobility was higher than modelled (synergism). On the other hand, no deviation was observed from independent action in algal tests. This study represents an important step to understand the interactions among pesticides detected previously in field monitoring studies of agricultural areas in “Lezíria do Tejo”, Portugal. Observed mixture toxicity was compared with predictions, calculated from the concentration response functions of chlorpyrifos and terbuthylazine at two realistic concentration ratios by applying the biologically relevant patterns in which deviations occurred. The assumption of these last yielded accurate predictions, although worst for the mixture ratio chlorpyrifos 0.17 and terbuthylazine 85 µg/L under consideration.

Keywords: Mixture, chlorpyrifos, terbuthylazine, *Daphnia magna*, *Pseudokirchneriella subcapitata*, toxicity

3.2. Introduction

Plant production has a very important place in the Community (EC, 2009). One of the most important ways of protecting plants and plant products against harmful organisms, including weeds, and of improving agricultural production is the use of plant protection products (PPP).

However, PPP can also have non-beneficial effects on plant production. Their use may involve risks and hazards for humans, animals and the environment, especially if placed on the market without having been officially tested and authorised and if incorrectly used. Since the use of pesticides in agriculture inevitably leads to exposure of non-target organisms (including humans), undesirable side-effects may occur on some species, communities or on ecosystems as a whole (van der Werf, 1996). Given the large amounts of pesticides applied globally and given the fact that they are designed to harm biota, there is a high potential for adverse environmental effects also on non-target communities (Schäfer *et al.*, 2011).

A correct risk assessment and management of chemicals is the basis for any chemical control and risk reduction measures and ultimately provides a basis for the sustainable use of substances (Backhaus *et al.*, 2010).

Since there is a multitude of chemicals in all environmental compartments and in exposed biota, it is impossible to test each and every imaginable mixture. These mixtures found in the environmental can be analysed using two different approaches: “top-down” and the “bottom-up”. The “top-down” approaches has based on complex mixtures extracted from biological tissue or environmental samples, trying to identify the individual compounds that contribute to the observed toxicity of the samples. On the other hand, “bottom-up” approaches predict the toxicity of a defined mixture, based on a priori knowledge of the chemical composition and toxicity of the mixture components (Pérez *et al.*, 2011).

This study is based on a site-specific exposure scenario that reflects the median load of pesticides in field drainage in Central-Portuguese agricultural areas after maize treatments in spring. In small agricultural streams (and other edge-of-field surface waters) the herbicide terbuthylazine and the insecticide chlorpyrifos are strongly dominating the toxicity at the same time and/or in sequence. This has been shown in field monitoring studies of agricultural areas in “Lezíria do Tejo”, Portugal (Silva *et al.*, 2015, Pereira, *in press*). Considering this, a “bottom-up” approach was chosen to assessed the derive patterns for toxicity response of the mixture.

In order to predict the mixture toxicity, two concepts were used, usually termed Concentration Addition (CA) (Loewe & Muischnekand, 1926) and Independent Action (IA) (Bliss, 1939); allow predicting the joint toxicity of chemicals in any mixture, using the single-substance toxicity information as input data and assuming non-interaction (Cedergreen *et al.*, 2013).

It has performed various investigations in order to analyse the power of both concepts in typical toxicological and ecotoxicological assays and for a range of different environmental chemicals. Several studies suggest that mixtures of contaminants that have the same mode of action (MOA) have a better fit to the CA model and the mixtures with different MOA tend to be best modeled by IA (Faust *et al.*, 2000; Altenburger *et al.*, 2003; Backhaus *et al.*, 2004b). However, can occur deviations from these reference models. There are three types of deviation that can occur: synergism/antagonism (S/A), dose level dependency (DL) and dose ratio dependency (DR). To model these deviations, it was used the MIXTOX model, proposed by Jonker *et al.* (2005). The size of the deviation from a reference model can also be analysed using indices and graphical methods. The called model deviation ratio (MDR) is usually used; this presents the ratio between the predicted effect concentration and observed effect concentration (Belden *et al.*, 2007).

In studies with binary mixtures, the dose-response surfaces are common used to visualise the possible interactions between two chemicals in a specific test system. These dose-response surfaces describe the entire concentration-response surface by designing an experiment that administer data for the full range of possible combinations between two chemicals. The graphical method normally used is the isobologram, where is possible to compare the predicted isobole to a concentration combination that cause a predefined effect (usually 50%) from each ratio, extrapolating these concentrations to the x/y plane (Cedergreen *et al.*, 2013).

The usual organization for this type of study involve 1) the determination of the toxicity of the mixtures components; 2) the use of these data to predict the mixture toxicity according to CA and/or IA models; and finally 3) the comparison of the experimentally observed mixture toxicity with the conceptual predictions (Cedergreen *et al.*, 2013).

These analyses will be done with well-known and established single-species assays using the green limnic microalgae *Pseudokirchneriella subcapita* and the microcrustacean *Daphnia magna*.

Few studies tested the effect of binary mixtures consisted of terbuthylazine and chlorpyrifos. Munkegaard *et al.* (2008) investigated whether interactions between the two pesticides can take place in the aquatic algae *P. subcapitata* and the aquatic macrophyte *Lemna minor*. Changes in swimming behaviour and the inhibition of AChE were related and synergistic patterns were observed when *Danio rerio* (Pérez *et al.*, 2013a) and *Chironomus riparius* larvae (Pérez *et al.*, 2013b) were exposed to chlorpyrifos mixtures containing atrazine and terbuthylazine. Apart of this, no mixture study with terbuthylazine or any *s*-triazine has, to our knowledge been done, in *D. magna*, using a ray design and response surface analyses.

Most studies are restricted specifically to “reference mixtures”, in which all the components were known to act either by an identical or by completely different molecular mechanisms of action (Backhaus & Faust, 2012; Junghans *et al.*, 2006). The experimental investigations on the predictive value of the concepts with environmentally realistic mixtures have been rarely conducted, hence, our knowledge on their performance under these circumstances is limited.

This study aims to overcome this limitation by comparatively analysing the predictive value of both concepts, CA and IA, for an example of a typical environmentally realistic mixture of pesticides.

In conclusion, the specific objectives of this study were to achieve more environmental realism in the scientific basis for forecasting risks and associated uncertainties of agricultural exposure situations by addressing the following questions:

- how relevant are combination effects of a binary mixture of pesticides (chlorpyrifos and terbuthylazine) that was found (or that co-occurred) in the aquatic environment under a maize field condition within Portugal, at different measured concentrations, on selected species (*P. subcapitata* and *D. magna*) of two different trophic levels (primary producers and primary consumers), and
- may the toxicity of such pesticide mixture be predictable from single substances toxicity data using the descriptive models, CA and IA, and if not, how the deviations from them are characterised using the MIXTOX model?

3.3. Materials and methods

3.3.1. Test-organisms and chemical compounds

The dormant eggs (*ephippia*) of the crustacean *D. magna* were hatched according to the Daphtoxkit F magna standard operation procedure (SOP, 2003), in a petri dish. The *ephippia* were incubate for 72h, at 20-22°C under continuous illumination of min. 6000 lux (light intensity at the top of the petri dish), with a “reconstituted” natural freshwater, according to the formula recommended by the International Standardization Organization (ISO, 1996), for the acute toxicity test with *D. magna*. After that, the eggs develop into neonates can then be used immediately for the toxicity tests.

The microalgae *P. subcapitata* was de-immobilized from algal beads and transferred into an adequate culturing medium (ISO, 2004) according to the Algaltoxkit F standard operation procedure (SOP, 2004).

In order to check the correct execution of the test procedures and the sensivity of the tests, a reference test with the chemical potassium dichromate ($K_2Cr_2O_7$) was performed for both.

The use of *ephippia* and algal beads, in Toxkits, allows to prevent the variability associated with recruitment/maintenance of live stocks in conventional biossays, keeping an identical sensitivity. Other advantages of these tests, when compared with the conventional, is that allows obtaining uniform exposure conditions (due to the biologically inert materials), obtaining a high uniform quality of the medium and minimizing the necessary equipment and the labour time.

The organisms were exposed to chlorpyrifos (with 99.0% of purity) and terbuthylazine (with 98.5% of purity) singly and as a binary mixture. The stock solutions were prepared in acetonitrile and stored at 6ºC. In order to execute the toxicity tests, at the different concentrations tested, stock solutions were dissolved in the culture medium, to each test-organism.

3.3.2. Immobility or mortality test with *D. magna*

The Daphtoxkit F magna test estimates the 48-h lethality/immobility of *D*. *magna* neonates (less than 24-h old) exposed to the test solutions. Each replicate consisted of five organisms per 10 mL of medium and was incubated in darkness at 20°C. The percentage of mortality was determined at the end of the 48-h exposure by quantifying the number of immobile organisms. A major condition for the validity of the test is that the number of dead + immobile organisms should not exceed 10% in the controls.

3.3.3. Growth inhibition test with *P. Subcapitata*

The Algaltoxkit F test estimates the 72-h growth of *P. subcapitata* in each test solution and all materials used were purchased with the kit. As the correspondent conventional assay (e.g., OECD, 2011), the algae concentration at the start of the test was approximately 1.10^6 cells mL^{-1} $replicate^{-1}$ culture, and all cultures were incubated at 24°C under continuous cool white fluorescent illumination (100 µE m^{-2} s^{-1}). Algal growth rate was determined by optical density measurements, at 670 nm in a Hitachi U-2000 spectrophotometer UV-Vis (Hitachi, Ltd., Tokyo, Japan), and expressed as the percentage of inhibition relatively to the control.

The test validation criteria, according to OECD 201 (OECD, 2011), indicates that the control growth rate must be at least 0.92 per day, which corresponds to an increase in cell density by a factor 16 in 72h.

In order to obtain the EC_{50s} value for D. magna for each pesticide, five concentrations were tested for chlorpyrifos and terbuthylazine with four replicates each; in addition, a control

with artificial culturing medium was also tested in quadruplicate. The same was done for the algae, but with three replicates for each concentration.

3.3.4. Experimental Design

The dose response surfaces for the binary pesticide mixtures were performed by using a ray design. This design consists of dose response curves of the two individual pesticides individually tested and a number of dose response curves of the pesticides mixed at predefined mixture ratios (Figure 1).

The number mixture ratios were selected according to the methodology presented in Pérez *et al.* (2011). The aim of this choice was to obtain a reliable coverage of effect of the two pesticides. In this article the nominal concentrations of the mixtures were calculated based on expected toxic strengths (TU) of: 0.375 (0.125 + 0.25; 0.25 + 0.125), 0.5 (0.125 + 0.375; 0.25 + 0.25; 0.375 + 0.125), 0.75 (0.125 + 0.625; 0.25 + 0.5; 0.375 + 0.375; 0.5 + 0.25; 0.625 + 0.125), 1 (0.125 + 0.875; 0.25 + 0.75; 0.375 + 0.625; 0.5 + 0.5; 0.625 + 0.375; 0.75 + 0.25; 0.875 + 0.125), 1.5 (0.75 + 0.75; 1 + 0.50; 0.50 + 1), 1.75 (1 + 0.75; 0.75 + 1) and 2 (1 + 1). With the EC_{50s} values for single exposures and these ratios, is possible to convert the TU_s into the concentrations that will be used to make the combination of chlorpyrifos and terbuthylazine.

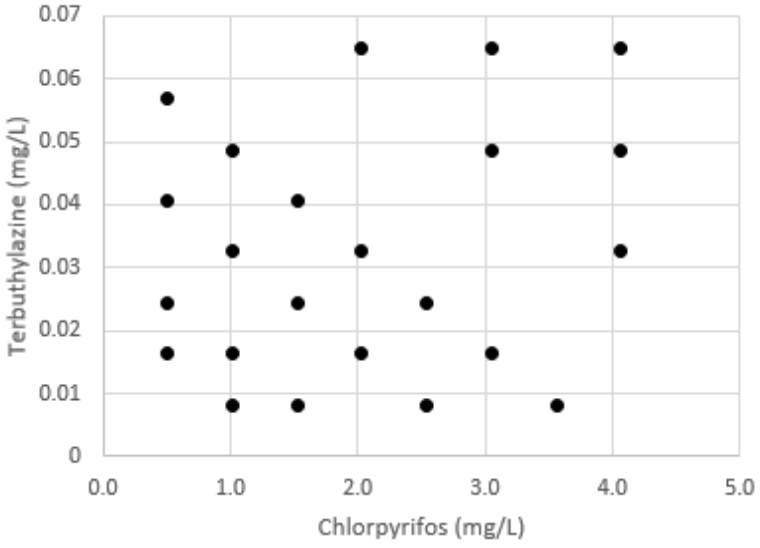

Figure 1 (Chapter 3). A fixed ray design of the combinations used for chlorpyrifos-terbuthylazine for *D. magna* and *P. subcapitata*.

3.3.5. Data analysis

The values of the EC_{50s}, and slope were derived, for single exposures, using the same dose-response-curve formula used within the MIXTOX model (Jonker *et al.*, 2005) namely a three-parameter logistic curve (Equation. 1), using the software SigmaPlot 13 (Systat, 2016).

$$Y_i = \frac{max}{1+\left(\frac{C_i}{EC_{50i}}\right)^{\beta_i}} \quad (1)$$

Where Y_i is the response of a given parameter at a concentration (C_i) of a chemical (i) that was calculated using the maximum response value (max) for that parameter, the EC_{50i}, and the slope (b_i) for the pesticide. The three-parameter logistic curve can be used for endpoints that decrease or increase with the increasing of the dose, depending on the slope (Jonker *et al.*, 2005).

To analyse the results obtained for the mixture exposures was used the MIXTOX model of Jonker *et al.* (2005), that compared the observed data with the expected mixture effects from both reference models. The second step was to extend both the CA and IA models, with deviation functions to describe synergistic/antagonistic interactions, dose-level, and dose-ratio dependency according to the methodology presented by Jonker *et al.* (2005). The parameters of the deviations were needed to build a nested framework. It was possible to fit the data to the models using the method of maximum likelihood and, as they are nested, the adjusted model can be statistically compared through likelihood testing (Neter *et al.*, 1996). When a statistically and more descriptive deviation model was identified, the effects pattern was directly deduced from the parameter values as described below, and in order assess the biological significance, the maximum deviation was calculated in effect concentration (CA) or effect level (IA) terms to (Loureiro *et al.*, 2010).

For the synergy/antagonism deviation model (S/A model), the extra parameter *a* can become negative or positive, respectively, for both reference models. When a=0, the S/A model reduces to the CA or IA. A second parameter b_{DL} con be included in addition to *a*, in order to generate the dose-level (DL) deviation model. In this case the value of *a* indicates the deviation at low doses (i.e., a>0=antagonism, and a<0=synergism) and the value of b_{DL} indicates at what dose level the deviation changes (i.e., from synergism to antagonism or vice versa). For CA/DL, the dose level where the deviation change occurs can be calculated using the follow expression: $1/b_{DL} \cdot EC_{50}$; e.g., $b_{DL}=1$ means that the switch occurs at the EC_{50} isobole. When $b_{DL}=0$, the equation reduces to the S/A model. If $b_{DL}<0$, the magnitude of synergism/antagonism (*a*) becomes dose-level dependent, but does not switch. In IA/DL deviation function, the switching can be estimated directly from $1/b_{DL}$; the switching occurs at

mixture doses that cause a specific level of effect. If b_{DL}=2, the switching occurs at doses where effect level is 50%. If b_{DL}=0, the deviation function again reduces to the S/A model. When b_{DL}<1, the magnitude of synergism/antagonism becomes response-dependent, but does not switch (Loureiro *et al.*, 2010).

For dose-ratio (DR)-dependency, again a second parameter b_{DR} is included in addition to *a*. The extra parameter b_{DR} express the dependency of the reference models on the composition of the mixture. In a binary mixture, antagonism can be observed where the toxicity of the mixture is caused mainly by toxicant 1, whereas synergism can be observed where the toxicity is mainly caused by toxicant 2. Therefore, the b_{DR} relates to the lead chemical of the mixture (i.e., the one mentioned and modelled first). In DR model, the parameter *a* quantifies the degree of antagonism (a>0) or synergism (a<0) and a significant b_{DR} quantifies the degree of reduced (b_{DR}>0) or increased (b_{DR}<0) toxicity due to the lead chemical. When *a* and b_{DR} have opposing signs, occurs a switch between antagonism and synergism within the response surface; whereas, if they have the same sign, the magnitude of the antagonism or synergism will vary with the ratio of chemicals, but not switch (Loureiro *et al.*, 2010).

Effects on the growth inhibition of *P. subcapitata*, and on the mortality and/or immobility of *D. magna* from exposures to mixtures with the pesticides chlorpyrifos and terbuthylazine were fit in a first step to the IA model as pesticides with different mode of actions, but the adjustment data was also performed for the model CA. Both models were tested in order to evaluate which model predict better the effects, and deviations evaluated.

The nested deviations were compared using the method of maximum likelihood and the best fit chosen using 0.05 as the significance level. In addition, the lowest residual sum of square (SS) was preferred when comparing conceptual models and deviations. For full details on the derivation of these deviation functions, refer to Jonker *et al.* (2005).

In the statistical tests, differences were considered significant when p-value ≤ 0.05. The statistical analysis were performed with the assistance of software SigmaPlot 13 (Systat, 2016).

3.4. Results and Discussion

3.4.1. Individual toxicity tests

The 48-h immobility with the single pesticides showed that the insecticide chlorpyrifos and the herbicide terbuthylazine were very toxic ($EC_{50} \leq 1$ mg/L; EC, 2001) at effective median concentrations to daphnid species, respectively. The 72-h growth inhibition tests results with the single exposures of the two pesticides showed that terbuthylazine was also very toxic to the microalgae, and chlorpyrifos was classified as toxic ($1 \leq EC_{50} \leq 10$ mg/L, EC, 2001).

The EC_{50} values obtained after the 48-h and 72 h of exposure were used to calculate the TU values for the mixture experimental setup. EC_{50} for 48 and 72 h obtained directly from the bioassays, as well the EC_{50} values in the literature are depicted in Table 1.

Table 1 (Chapter 3). EC_{50} values in the present study and literature.

Pesticides	*D. magna*		*P. subcapitata*	
	In this study	**Literature**	**In this study**	**Literature**
CPF	0.24 (0.20-0.30)[1] µg/L	0.12-9.07 µg/L[2]; 0.1 µg/L[7]	4.067 (2.780-7.880)[1] mg/L	6.6-53 mg/L[5]; 480 µg/L[7]
TBZ	0.95 (0.80-1.130)[1] mg/L	5-21.2 mg/L[3]; ≥ 69.3 mg/L[4]; 21.2 mg/L[7]	65 (46-100)[1] µg/L	24-55 µg/L[6]; 0.016 mg/L[4]; 1.2 µg/L[7]

[1]EC_{50} values with 95% confidence interval
[2]ECOTOX, 2016; Kikuchi *et al.*, 2000; Gaizick *et al.*, 2001; Palma *et al.*, 2008; Antunes *et al.*, 2010; Rubach *et al.*, 2011; Liu *et al.*, 2012
[3]Marchini *et al.*, 1988; ECOTOX, 2016
[4]McBean, C., 2012
[5]Antunes *et al.*, 2010
[6]Okamura *et al.*, 2000; Cedergreen and Streinig, 2005; Pérez *et al.*, 2011
[7]IUPAC, 2016

The EC_{50} values for crustacean *D. magna* are in the same order of magnitude as those reported in studies present in the table above for the chlorpyrifos. For terbuthylazine the value calculated in this study is lower than the literature, being more sensitive. The microalgae showed to have results in the same order of magnitude than in literature.

3.4.2. Binary mixture toxicity tests

The results obtained from fitting the data to the MIXTOX model are showed in Table 2 and 3, for immobilization and growth inhibition tests, respectively. The most important values are the SS, that quantify the model fit, and the value of $p(\chi^2)$, which indicates the significance of the deviations that can occur from the reference models.

For the fit of the CA model to the binary mixture data, for the immobilization test of *D. magna*, it was obtained an SS value of 218.1 (r^2 = 0.605; Figure 2). Adding the extra parameter *a*, to describe synergism/antagonism, the SS value decreased a little, but not significantly ($p[\chi^2]$ = 0.176), so the data showed no indication of synergism/antagonism. Adding to parameter *a*, parameters b_{DL} and b_{TBZ} the SS value decreased, but again not in significantly (both $p[\chi^2] > 0.05$), which indicates that there are no deviations from the reference model (Table 2). This is shown in the isobole diagram of the Figure 3A.

Comparing this data to de IA model, the SS value obtained was 228.7 (r^2 = 0.586). Adding the parameter *a* to the IA model, the SS value decreased slightly, not significantly ($p[\chi^2]$ = 0.569). Adding parameters *a* and b_{TBZ} through the model, the SS decreased a little but again not significantly ($p[\chi^2]$ = 0.404). However, adding parameters *a* and b_{DL} the SS value decreased significantly to 213.6 (r^2 = 0.613; $p[\chi^2]$ = 0.0005; Figure 2), and a dose level-dependent deviation from independent action was concluded. The positive value of *a* (3.529) in the deviation model, indicates that occurs antagonism at low dose levels and synergism at high dose levels. Parameter b_{DL} being positive and approximately 2, indicating a shift between antagonism and synergism at the EC_{50} value (1/2=0.5) (Table 2). This is shown in the isobole diagram of the Figure 3B. The statistical analyses revealed that the DL deviation model explain more variance in the data than S/A model (χ^2 = 14.871; $p[\chi^2]$ = 0.0001).

Table 2 (Chapter 3). Summary of the analysis of the effect of the mixture on *D. magna*, using the MIXTOX model.

	Concentration Addition				**Independent Action**			
	Reference	**S/A**	**DR**	**DL**	**Reference**	**S/A**	**DR**	**DL**
μ_{max}	0.91	0.91	0.91	0.91	0.90	0.90	0.90	0.90
β_{TBZ}	3.9	3.9	3.9	3.9	3.0	3.0	3.0	3.0
β_{CPF}	5.3	5.3	5.3	5.3	3.0	3.0	3.0	3.0
EC_{50TBZ}	1.18	1.18	1.18	1.18	0.90	0.90	0.90	0.90
EC_{50CPF}	0.00025	0.00025	0.00025	0.00025	0.0002	0.0002	0.0002	0.0002
a	NA	0.209	-0.343	-0.00081	NA	-0.212	-1.496	3.529
b_{DL}	NA	NA	NA	199.553	NA	NA	NA	2.152
b_{TBZ}	NA	NA	1.266	NA	NA	NA	2.757	NA
SS	218.1	216.3	214.6	216.7	228.7	228.4	226.9	213.6
χ^2	334.16	NA	NA	NA	NA	0.324	NA	15.195
df	NA	1	2	2	NA	1	2	2
$p(\chi^2)$	4.62×10^{-71}	0.176	0.173	0.484	NA	0.569	0.404	0.0005

Equations used to derive these results are detailed in Jonker *et al.* (2005).

μmax is the control response (maximum immobility); β is the slope of the individual dose-response; EC_{50} is the median effect concentration (mg/L); *a*, b_{DL}, and b_{TBZ} are parameters in the deviation functions; SS is the residuals sum of squares; χ^2 is the test statistics; df is the degrees of freedom; and $p(\chi^2)$ indicates the outcome of the likelihood ratio test (significance level $p < 0.05$). The abbreviation NA means quantity is not applicable.

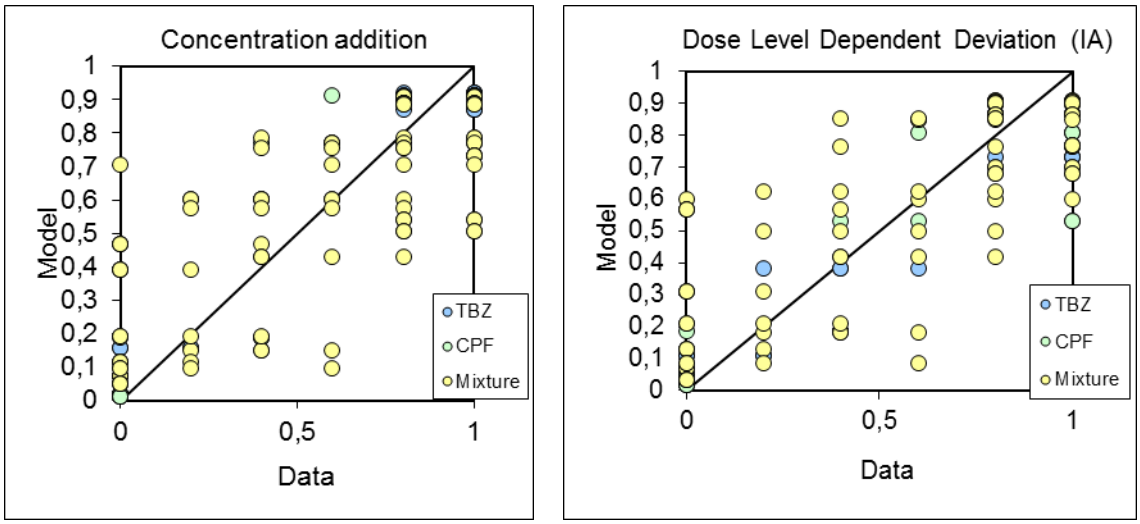

Figure 2 (Chapter 3). Relationship between observed data from *D. magna* exposures and the modelled values. Left column: data vs modelled values using the CA reference model; right column: data vs modelled values using the IA model deviation.

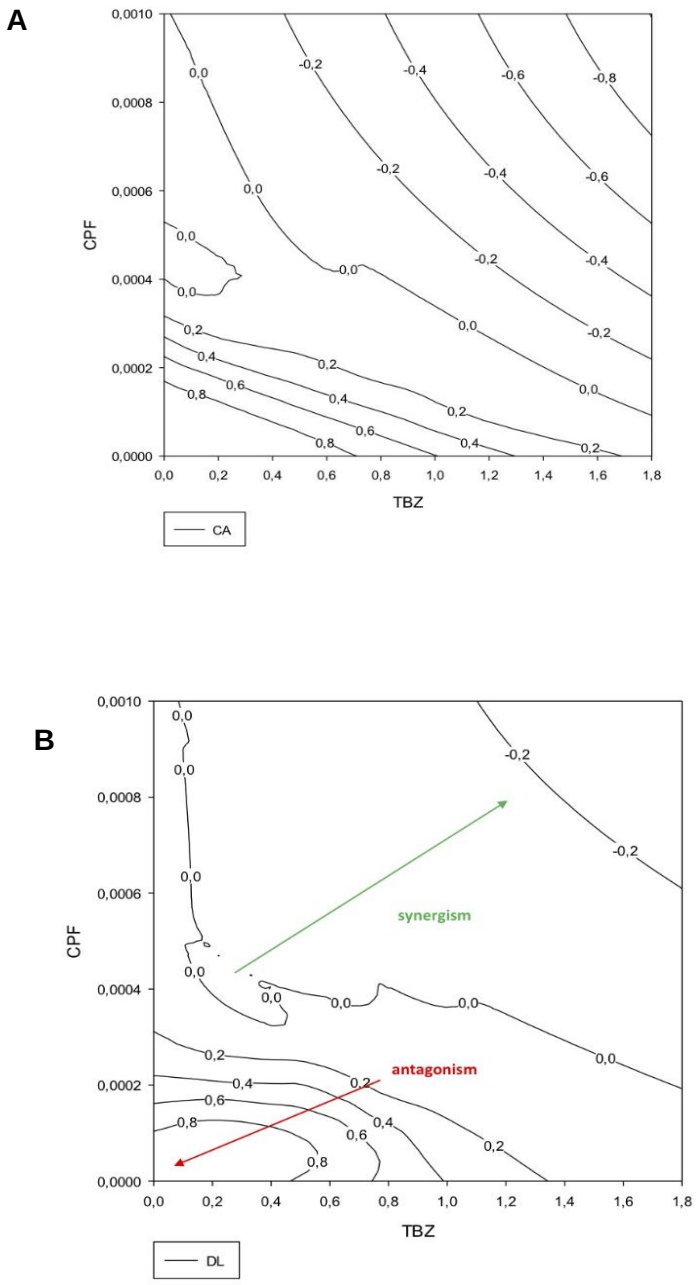

Figure 3 (Chapter 3). Concentration-response relationship for the binary mixture of terbuthylazine and chlorpyrifos (2D isobolic surfaces) of the survival of D. magna: (A) Concentration Addition model fits, (B) Dose-level deviation after the Independent Action model fits.

During this study the main question was how well the reference models predict the joint effects of the mixture chosen, for both organism, and how the conceptual models becomes useful having *a priori* knowledge of the MOA of both pesticides. It is known that chlorpyrifos and terbuthylazine have a different molecular MOA. Theoretically, the IA model should be the preferred reference model. For D. *magna*, the higher proportions of the total variation explained by both reference models fits of 60.5% versus 58.6% to CA and IA model,

respectively. This comparison between reference models showed that CA described a slightly higher proportion of the total variance than the IA, contrary to what would be predicted. However, a dose level-dependency was detected in the fit of the IA model that justified 61.3% of the total variance, slightly higher than the CA model.

In the study of Loureiro *et al.* (2010) with *D. magna*, the combined effects of pesticides and nickel were adjustable both to the IA and CA models, however the IA model can be chosen since the modes of action are dissimilar. Loureiro *et al.* (2009) studied other crustacean, where *Porcellionides pruinosus* exposed to atrazine and dimethoate (an organophosphate insecticide) showed a significant dose level dependent deviation from the IA model, showing antagonism at low dose levels and synergism at high dose levels, with no deviation for CA model.

Other studies provided examples where IA is not the best model to explain the data, as shifts for synergism and/or antagonism might occur depending on the dominant chemical present. Synergistic deviations from the conceptual models of mixtures have been frequently found in previous studies with invertebrates, showing that there may be an interaction between chemicals rather than an additive or independent response. Species such as *Chironomus tentans*, *Hyalella azteca* and *Ceriodaphnia dubia* exposed to atrazine and organophosphate insecticide mixtures have shown greater than additive toxicity (PapeLindstrom & Lydy, 1997; Anderson & Lydy, 2002; Belden & Lydy, 2000; Banks *et al.*, 2005; Schuler *et al.*, 2005; Jin-Clark *et al.*, 2002; Lydy & Austin, 2004; Trimble & Lydy, 2006). The combined effects of dimethoate and atrazine showed mainly synergistic patterns in *Folsomia candida* (Amorim *et al.*, 2011). A standard Organization for Economic Cooperation and Development (OECD) filter paper test was used to assess the acute toxicity of chlorpyrifos, atrazine, cyanazine, and simazine to the earthworm *Eisenia fetida*. Atrazine and cyanazine also increased the toxicity of chlorpyrifos 7.9- and 2.2-fold, respectively. However, simazine caused no toxicity to the worms and did not affect chlorpyrifos toxicity in binary mixture experiments. Possible mechanisms for the greater-than-additive toxicity for the binary combinations of atrazine and cyanazine with chlorpyrifos were investigated, including changes in uptake and biotransformation rates of chlorpyrifos in the presence of atrazine. Uptake of chlorpyrifos into the worms decreased slightly when atrazine was present in the system, therefore eliminating increased uptake as a possible explanation for the increased toxicity. Body residue analysis of worms indicated increased metabolite formation, suggesting the greater-than-additive response may be due to increased biotransformation to more toxic oxon metabolites (Lydy & Linck, 2003). Yang *et al.* (2015) showed that the binary mixture of chlorpyrifos and atrazine was antagonistic toward *E. fetida* at all f_a levels in an artificial soil test. For the *Enchytraeus albidus* the exposure to the mixture atrazine and dimethoate showed a significant deviation from the IA model fit for antagonism (Loureiro *et*

al., 2009). Wacksman *et al.* (2006) examined the interactions between atrazine and chlorpyrifos in four aquatic vertebrate species, and the presence of atrazine at 1.000 µg/L resulted in a significant increase in the acute toxicity of chlorpyrifos in the African clawed frog (*Xenopus laevis*). For the fish *Pimephales promelas* a lack of a clear toxicity pattern was observed, since that some bioassays results showed greater than additive toxicity, while others showed an additive response. In the other organisms studied (*Lepomis macrochirus* and *Rana clamitans*), no effect of atrazine on chlorpyrifos toxicity was observed (Wacksman *et al.*, 2006). Xing *et al.* (2015) results also suggest that exposure to atrazine, chlorpyrifos or their combination promotes oxidative stress and autophagic responses in the brain of the common carp (*Cyprinus carpio L.*).

A study with *Danio rerio* in early-life stages, using the binary combinations of atrazine and terbuthylazine with chlorpyrifos, suggest that the s-triazine herbicides potentiated the chlorpyrifos toxicity. Changes in swimming behaviour and the inhibition of AChE were related and synergistic patterns were observed when zebrafish larvae were exposed to the binary mixtures. The increased of the chlorpyrifos toxicity with the presence of these herbicides it happens possibly due to the effect of s-triazines to accelerated the transformation of chlorpyrifos in its oxon form, increasing therefore toxicity by inhibiting AChE activity (Pérez *et al.*, 2013a). Pérez *et al.* (2013b), also studied this mixture in the *Chironomus riparius* larvae, when combined with both s-triazine herbicides, chlorpyrifos toxicity was enhanced by approximately 2-fold when tested in a binary mixture experimental setup, at the 50% effective concentration levels. Atrazine and terbuthylazine are not effective inhibitors of AChE, however they potentiate chlorpyrifos toxicity; both s-triazine herbicides at 200 µg/L increased the inhibition of the AChE activity by 7 and 8-fold, respectively.

These patterns were not coincident with the ones described here, showing dose-level deviations (antagonism at low concentrations and synergism at high concentrations) for the crustacean *D. magna*. Such differences could be due to species and endpoint specificity. Only the study with *Porcellionides pruinosus* (Loureiro *et al.*, 2009) presents similar deviation patterns to our study.

To evaluate the joint effects of the mixture on the growth of the algae *P. subcapitata*, both reference models, CA and IA, were also used. In the fit of the CA model to the data the SS value obtained was 7.281 ($r^2 = 0.64$; Figure 4). With the adding of the parameters *a* and b the decrease of the SS value was not significant in either case, so was concluded that the data fits to the CA model ($p \leq 0.05$) (Table 3). This is shown in the isobole diagram of the Figure 5A.

In the IA model, the fit provided a SS value of 8.098 ($r^2 = 0.60$; Figure 4). Again, the adding of the parameters *a* and b do not provided a significant decreased of the SS values,

concluding that do not occur deviations from this model. Therefore, the data fits to both reference models, however the CA models explains slightly better the proportion of the total variance than the IA model (Table 3). This is shown in the isobole diagram of the Figure 5B.

Table 3 (Chapter 3). Summary of the analysis of the effect of the mixture on *P. subcapitata*, using the MIXTOX model.

	Concentration Addition				Independent Action			
	Reference	**S/A**	**DR**	**DL**	**Reference**	**S/A**	**DR**	**DL**
μ_{max}	0.772	0.771	0.769	0.772	0.737	0.776	0.774	0.764
β_{TBZ}	0.833	0.826	0.808	0.833	0.896	0.764	0,764	1.021
β_{CPF}	0.765	0.787	0.808	0.765	0.732	0.641	0.641	1.092
EC_{50TBZ}	0.056	0.053	0.056	0.056	0.084	0.049	0.049	0.058
EC_{50CPF}	3.809	3.55	3.81	3.809	6.154	4.567	4.567	3.764
a	NA	0.420	0	0	NA	1.367	0.079	-3.951
b_{DL}	NA	NA	NA	1	NA	NA	NA	2.073
b_{TBZ}	NA	NA	0.919	NA	NA	NA	2.233	NA
SS	7.281	7.26	7.26	7.28	8.098	7.62	7.58	6.3
χ^2	13.0	NA	NA	NA	12.187	NA	NA	NA
df	NA	1	2	2	NA	1	2	2
$P(\chi^2)$	0.011	0.893	0.988	1	0.016	0.490	0.772	0.406

Equations used to derive these results are detailed in Jonker *et al.* (2005).

μmax is the control response (growth rate); β is the slope of the individual dose-response; EC_{50} is the median effect concentration (mg/L); *a*, b_{DL}, and b_{TBZ} are parameters in the deviation functions; SS is the residuals sum of squares; χ^2 is the test statistics; df is the degrees of freedom; and $p(\chi^2)$ indicates the outcome of the likelihood ratio test (significance level $p < 0.05$). The abbreviation NA means quantity is not applicable.

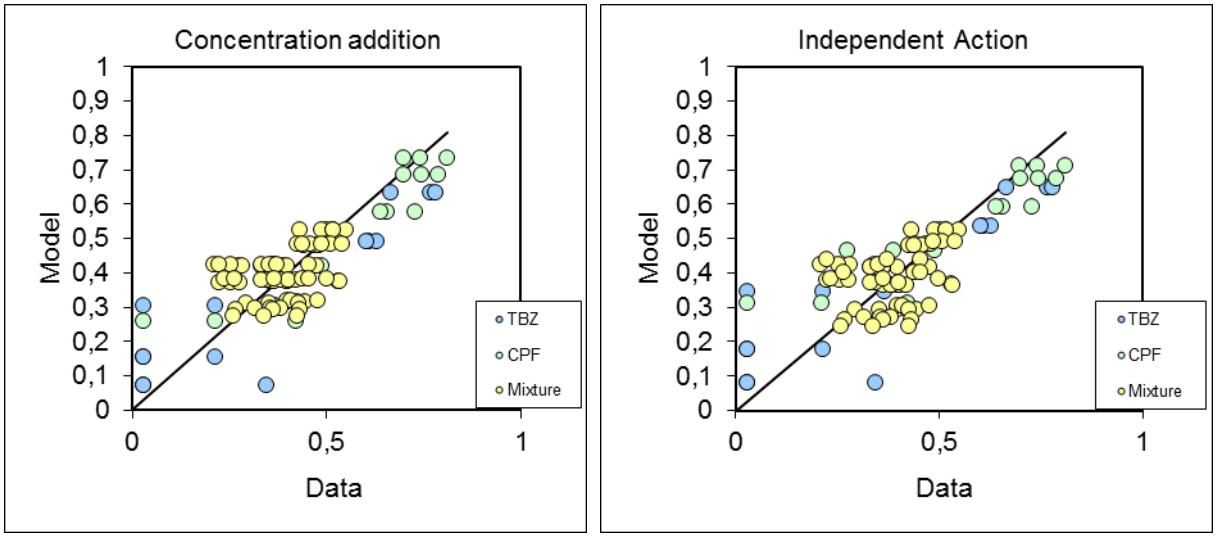

Figure 4 (Chapter 3). Relationship between observed data from *P. subcapitata* exposures and the modelled values. Left column: data vs modelled values using the CA reference model; right column: data vs modelled values using the IA reference model.

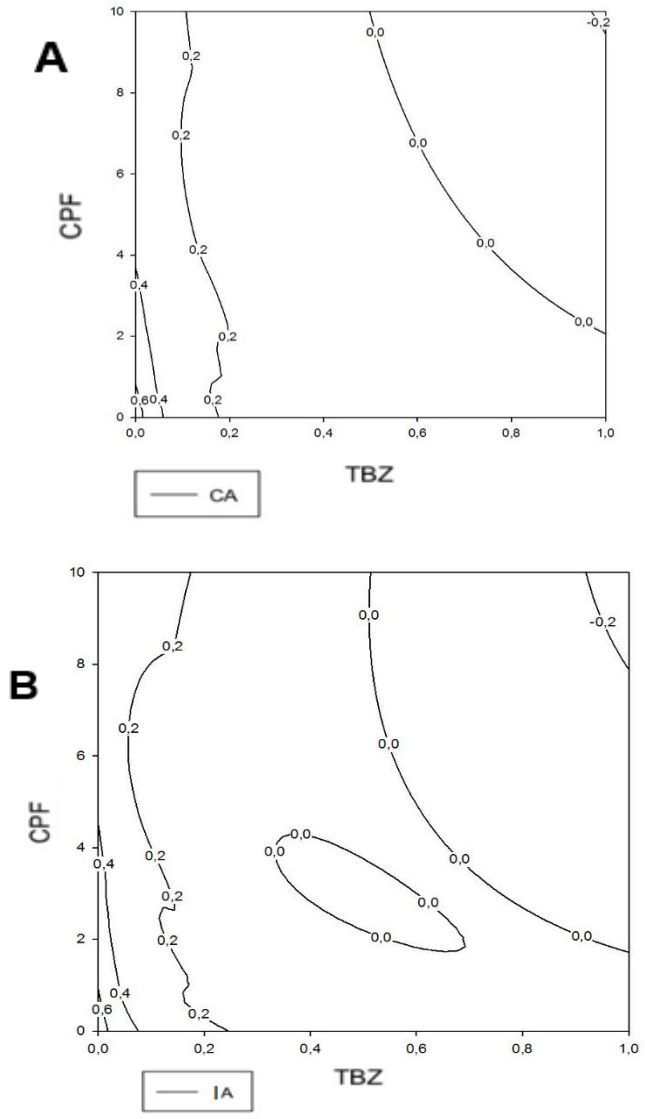

Figure 5 (Chapter 3). Concentration-response relationship for the binary mixture of terbuthylazine and chlorpyrifos (2D isobolic surfaces) of the growth of *P. subcapitata*: (A) Concentration Addition model fits, (B) Independent Action model fits.

Regarding chemicals with different molecular target sites, previous studies with the unicellular green freshwater algae *Scenedemus vacuolatos* demonstrated that the IA conceptual model presented a better prediction when compared to the CA model when testing the mixtures of 16 biocides (Faust *et al.*, 2003). The mixture toxicity of different pollutants with unclear modes of action was also accurately predicted by IA at individual NOECs on the growth of the algae *S. vacuolatus* (Walter *et al.*, 2002).

In addition, Backhaus *et al.* (2004a) employed this IA model to predict the toxicity of six dissimilarly acting substances on the natural algae communities.

In the study performed by DeLourenzo & Serrano (2003), the mixture of atrazine and chlorpyrifos had additive toxicity to Dunaliella tertiolecta (Chlorophyta, green algae). Belden

& Lydy (2000) however, found that atrazine and chlorpyrifos in mixture exhibited synergistic toxicity to the midge larvae Chironomus tentans. Atrazine was found to increase the biotransformation of the organophosphate compound, converting it into a more toxic metabolite. While this mechanism enables atrazine and chlorpyrifos to be synergistic in mixture to an invertebrate species, there is no comparable mechanism for chlorpyrifos toxicity in phytoplankton.

The study with the test organisms *P. subcapitata* and *Lemna minor* shows no indications of synergistic interactions between the tested pesticides, confirming the applicability of CA as a reference model predicting mixture effects of pesticides for aquatic plants and algae (Munkegaard *et al.*, 2008). These pesticides in mixture displayed additive toxicity, which are in accordance with the results of our study.

3.4.3. Toxicity from the agricultural exposure scenario

When chlorpyrifos and terbuthylazine are present at their measured concentrations in field surface waters, the mobility on *D. magna* was affected by 45% (mixture 1: chlorpyrifos 0.17 and terbuthylazine 8.5 µg/L) and 75% (mixture 2: chlorpyrifos 0.17 and terbuthylazine 85 µg/L). The mixture 1 observation is in good agreement with the prediction derived from independent action with a dose level-dependent deviation (43%), and mixture 2 is higher than predicted by the deviation pattern (dose level dependence) from this reference model (41%).

The two pesticides, chlorpyrifos and terbuthylazine, were shown to cause a total effect on *P. subcapitata* of 31% (mixture 1) and 88% (mixture 2). Fairly good compliance with the effect predicted by concentration addition and independent action (35% and 34%, respectively) demonstrates a high predictive power of both concepts for mixture 1, although observed mixture toxicity and both predictions differed by a factor of 1.4 for mixture 2 with 85 µg/L terbuthylazine.

3.5. Conclusion

Our study supports the usefulness of the reference models concentration addition and independent action and their possible deviations to ecological risk assessment of relevant pesticide mixtures in aquatic ecosystems.

Although with only two test species, this study restates the differences in species sensitivity in ecotoxicological approaches, and alerts for the lack of information that single chemical exposures give to the actual needs of ecological risk assessment procedures.

Regarding the exposure to the binary mixture of chlorpyrifos and terbuthylazine we expected that these two chemicals act dissimilarly, thus we first use the IA conceptual model. Although the algae data did not deviate significantly from the conceptual model IA, for the

daphnids a significant deviation from the IA model with antagonism at low dose levels and synergism at high dose levels was observed. The CA model can be chosen for algae since it is the model recommended for risk assessment purposes as it most often gives the most conservative prediction of joint effects (Faust & Scholze, 2004).

Observed mixture toxicity for the real exposure concentrations was compared with predictions, calculated from the concentration response functions of chlorpyrifos and terbuthylazine at two realistic concentration ratios by applying the biologically relevant patterns in which deviations occurred. The assumption of these last yielded accurate predictions, although worst for the mixture ratio chlorpyrifos 0.17 and terbuthylazine 85 µg/L under consideration.

In final conlusion, daphnids proved to be fairly well described by the models used in the study, already described by Jonker *et al.* (2005).

On the other hand, *P. subcapitata* proved to be very robust in terms of substances affecting metabolism in other organism, being less susceptible to synergy, this hypothesis is supported by the literature (Munkegaard *et al.*, 2008). The algae is also well described by the models.

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